POLYPHENOL OXIDASE ACTIVITY AND PIGMENTATION IN SNAIL TISSUES [1]

JACK W. DAUGHERTY

The Rice Institute, Houston, Texas and The U. S. Army Tropical Research Medical Laboratory, San Juan, Puerto Rico

Relatively little has been done to elucidate the mechanisms of intermediary metabolism in molluscs despite their recognized medical and economic importance (von Brand, 1952). A knowledge of such mechanisms would seem to be an essential preliminary to any rational investigation of these forms as parasite vectors or any logical attempt toward their control. In addition, comparative information concerning metabolically important reactions in different animals is a valuable aid to our understanding of the patterns of animal metabolism in general.

As part of an over-all study of snail physiology and biochemistry various aspects of the functioning of the midgut gland (hepatopancreas) of certain fresh water snails have been investigated. During one phase of this program a study was made of the mechanisms thought to be responsible for the presence of the reasonably large amount of pigment normally found in this gland in the fresh water operculate snail, *Ceratoides cornvarietis* (Linné, 1758). The deposition of melanin pigments in various tissues is a characteristic activity in many organisms, but the function, if any, of such pigments is frequently obscure. Since the pigmentation is the normal result of metabolism in the liver of the snail and since it has been suggested that the oxidative components of the series of chemical transformations resulting in melanin formation may be linked to the electron transfer systems of certain aerobic dehydrogenases (Baldwin, 1952) it was felt that a study of the function of snail liver must include some references to these reactions. The present report deals with some of the characteristics of the polyphenol oxidases in snail liver and their probable connection with melanin formation.

Methods

The fresh water snail, *Ceratoides cornvarietis,* used in the present study was initially obtained from a stream near Rio Piedras, Puerto Rico. The animals used in the present study were raised in this laboratory from the original stock. This snail is not a vector for any known medically important parasite, but was chosen for the initial studies in snail physiology because of its large size, abundance, hardiness in the laboratory, and, importantly, because of the fact that in this snail, by careful dissection, the gonads could be fairly well separated from the midgut gland. It was expected that the results obtained and techniques developed in the study of this snail would allow a more effective approach to the later study of more medically

[1] This study was supported (in part) by a research contract with the Office of the Surgeon General, U. S. Army and (in part) by a grant, No. E-374, from the National Institutes of Health, Public Health Service.

important forms. The snails were kept in the laboratory in constantly aerated pond and well water aquaria. They were not starved (Baldwin, 1938) before experimental use as no significant improvement in the consistency of results was obtained in this manner.

The assays for enzyme activity were carried out on cell-free homogenates (Potter and Elvehjem, 1936) prepared in cold, glass-distilled water from tissue samples carefully dissected from large mature snails. The use of varying salinities as suspending media failed to significantly alter the results. In the preliminary experiments the tissues were segregated according to the sex of the animal, but when no fundamental differences were observed this procedure was abandoned. The homogenates were uniformly centrifuged at low speed ($600 \times g$) to remove the major part of the heavy inert pigment before being diluted to a final 5% concentration. This dilution effectively prevented any oxygen consumption by endogenous metabolism. All of the foregoing procedures were carried out at 4° C. and the reaction flasks were kept thoroughly chilled until placed in the water bath. The reaction mixtures were assayed in Warburg flasks in an atmosphere of air at 25° C.

TABLE I

Per cent dry weight, total nitrogen, and the ether-soluble fraction of snail liver

% dry wt.		N_2 mg./gm. dry wt.		Lipid mg./gm. dry wt.	
Male	Female	Male	Female	Male	Female
27.4	25.6	74.8	68.2	164	230
27.0	28.9	75.2	67.4	144	209
27.0	27.1	72.9	68.2	151	214
28.1	28.2	73.4	69.1	153	198
27.2	26.8	72.8	66.9	167	207
27.0	26.9	72.9	67.0	172	222
26.9	28.1	74.3	68.2	154	214

Suitable controls were included in each run. Initial runs were made to insure optimum conditions in regard to enzyme concentration, substrate concentration, and pH. Dry weight determinations were made to check against differences in individual tissue samples as were total nitrogen determinations as a crude test for total protein (micro-Kjeldahl) and fat determinations (ether-soluble weight loss method).

Results and Discussion

During the course of the assays for specific enzyme activity periodic checks gave the results on dry weight, total nitrogen, and the ether-soluble fraction which are summarized in Table I. Although both male and female livers have *ca.* 27% dry material, the male liver contained somewhat more nitrogen and less ether-soluble material. In dissecting out the female liver some difficulty was encountered in completely freeing the liver from extraneous tissues. The differences in the nitrogen and fat data between the sexes, therefore, are possibly a reflection of the difference in the closeness of the anatomical relationship of the liver and gonad. Apparently, however, this difference in the tissue samples was not significant from

TABLE II

Influence of enzyme concentration, substrate concentration (catechol), and pH on the activity of polyphenol oxidase activity in snail liver homogenates. All but the experimental substances were at optimum concentration

Enzyme concentration (mgm. snail liver, wet wt.)			5	10	25	30
Q_{O_2}			26 (23–29)	56 (54–58)	108 (99–115)	111 (101–121)
Substrate concentration (catechol in micromols)			10	20	50	60
Q_{O_2}			25 (23–29)	42 (38–47)	99 (95–110)	103 (98–108)
pH	6.8	7.2	7.6	8.0	8.4	8.8
Q_{O_2}	41 (35–52)	73 (67–79)	92 (86–99)	106 (97–111)	91 (87–96)	62 (53–71)

the standpoint of the enzyme activity, since no variation between sexes in this regard was obtained from the assays.

In Table II are given the results of studies on the influence of enzyme and substrate concentration and the pH on the activity of the snail liver polyphenol (catechol) oxidase activity. Optimum conditions were obtained with a reaction mixture containing 25 mgm., wet weight, of cell-free homogenate, 50 micromols of substrate (catechol), and 0.033 *M*, final, potassium phosphate buffer at a pH of 8.0. The addition of neither methylene blue, diphosphopyridine dinucleotide, nor cytochrome *c* to the reaction mixture to serve as a hydrogen carrier was of any benefit to this system. From this it would seem that the system is an aerobic oxidase rather than an aerobic dehydrogenase. The replacement of the air in the reaction system with 100% oxygen was also of no significance in promoting higher activity. The enzyme was found to exhibit a marked stability for several hours at room temperature and it was still highly active after refrigeration for 96 hours.

TABLE III

Activity of the monohydric and polyhydric phenol oxidases in snail liver homogenates. Reaction flask contained 25 mgm. (wet weight) liver homogenate, 0.1 M pH 8.0 phosphate buffer, 50 micro. mol. substrate, 1 ml. 20% NaOH in center well. Each figure is the average of closely approximated duplicates

Substrate	Q_{O_2}					
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
p-Cresol	0	0	0	0	0	0
Phenol	16	0	0	0	0	0
Tyrosine	0	0	9	0	0	0
Guaiacal	6	0	21	0	0	0
Catechol	104	107	107	106	110	99
Pyrogallol	142	141	144	145	132	134
Adrenaline	72	79	75	69	72	73

In the frozen or lyophilized state it was stable up to six months. In this regard the enzyme resembles the tyrosinase of earlier workers (Lardy, 1949).

However, the enzyme system in snail liver was observed to seemingly possess a generic specificity for polyhydric phenols (Table III). Negligible activity was

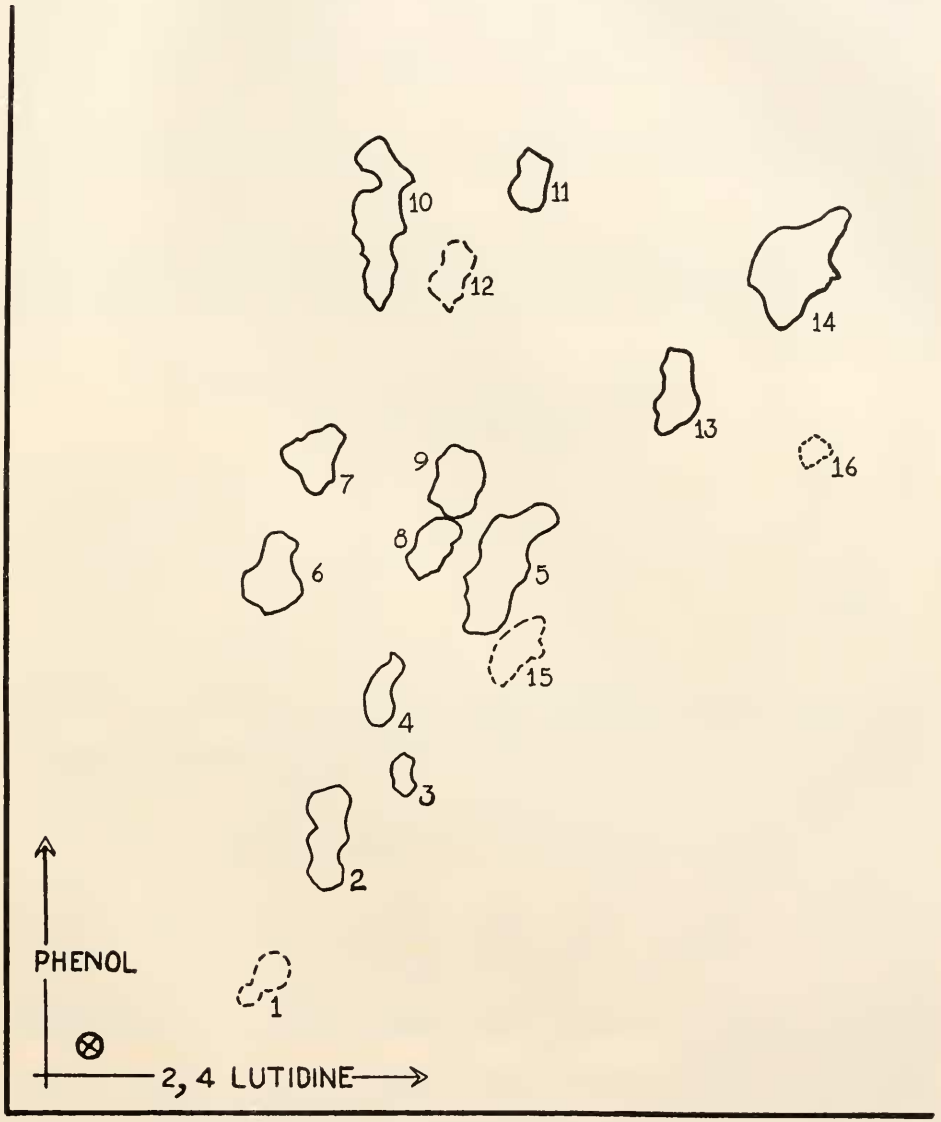

FIGURE 1. Two-dimensional composite chromatogram of the liver from *C. cornvarietis* extracted for free amino acids. Broken lines indicate relatively low concentration. 1—aspartic acid; 2—glutamic acid; 3—serine; 4—glycine; 5—alanine; 6—lysine; 7—arginine; 8—citrulline and/or glutamine; 9—β-alanine; 10—unidentified polypeptid; 11—proline; 12—unidentified; 13—valine; 14—methionine and leucine; 15—threonine; 16—unidentified trace.

occasionally obtained on p-cresol, phenol, tyrosine, or guaiacol substrates whereas catechol, adrenaline, and, to an even greater extent, pyrogallol provided relatively excellent substrates for the system. From these data it appears that the enzyme which oxidizes the polyhydric phenols in snail liver is not identical with that reported for certain plant tissues (Nelson and Dawson, 1944). The enzyme described by these earlier workers (tyrosinase) was active against both polyhydric phenols and monohydric phenols, although the activity against the latter was usually somewhat unstable. It would seem that the enzyme described herein more closely approximates the catecholase studied by Graubard (1939) which also attacks only polyphenols. However, in this regard it should be remarked that the activity of tyrosinase against catechol has been reported as being *ca.* 124 times that against tyrosine (Kubowitz, 1938). Therefore, it was considered possible that the amount of tissue used in the major part of the present study was insufficient to demonstrate the action against tyrosine and other monophenols, especially since the maximum polyphenol oxidase activity measured was considerably less than that demonstrable on potato and mushroom preparations. In testing this, however, no combination

TABLE IV

Survey of polyphenol oxidase (catecholase) activity in snail tissues. Reaction mixture as in Table III

Tissue	Q_{O_2}		
	No. 1	No. 2	No. 3
Liver	105	108	107
Foot	29	22	20
Mantle	68	64	55

of reactants was found which at any time permitted anything but periodic and highly insignificant activity with monophenol substrates.

In view of the demonstrated inactivity of snail tissues against monophenols, liver extracts were qualitatively analyzed for free amino acids, using two-dimensional ascending paper partition chromatography with phenol and 2, 4-lutidine as solvents, to determine the presence and metabolic availability of tyrosine as a substrate for enzyme activity *in vivo*. Glutamic acid, aspartic acid, valine, β-alanine, serine, glycine, arginine, lysine, alanine, proline, leucine, methionine, citrulline and/or glutamine were readily identifiable in good quantity but at no time was there detected any appreciable amount of tyrosine (Fig. 1). This apparent absence of tyrosine is consistent with the failure of the tissue preparations to oxidize monohydric phenols and the failure of the intact snail to produce pigment from tyrosine substrates.

Although maintaining the snails in the presence of catechol for 7–14 days prior to the enzyme assays failed to elicit any increased activity, it was observed that a decline in enzyme activity accompanied the decolorization of the tissues of snails kept for several weeks in tap water without food. This observation seems to associate the enzyme action with the pigmentation of the snail *in vivo,* particularly since other metabolic systems not concerned with melanin production (respiratory

enzymes, transaminase, and the amino acid oxidases) were not similarly affected (current unpublished results).

Further evidence of an association of the polyphenol oxidase activity with pigmentation was given as the result of enzyme studies on other tissues of the snail (Table IV). Both the mantle and the foot, in order, showed some activity but far less than that described above for the liver. It was interesting to note that the level of activity in the three tissues corresponded roughly to the degree of pigmentation, the foot in this snail being relatively free of coloration.

Acknowledgment is made to Dr. Harold Harry, U. S. Army Tropical Research Medical Laboratory, San Juan, Puerto Rico, for the initial collecting of the snails used in this study.

Summary

A study was made on the polyphenol oxidase activity of snail tissues. The liver showed higher activity than either the mantle or foot; however, even in this organ only polyhydric phenols were effectively oxidized. No consistent activity on monophenol substrates was found. The possible association of the polyphenol oxidase system to the heavy pigmentations in the liver was discussed.

LITERATURE CITED

Baldwin, E., 1938. On the respiratory metabolism of *Helix pomatia*. *Biochem. J.*, **32**: 1225–1237.

Baldwin, E., 1952. Dynamic aspects of biochemistry. The University Press, Cambridge, England.

Graubard, M., 1939. A comparative study of some oxidases and peroxidase. *Enzymologia*, **5**: 332–346.

Kubowitz, F., 1938. Spaltung und Resynthese der Polyphenoloxydase und des Hemocyanins. *Biochem. Zeitschr.*, **299**: 32–57.

Lardy, H. A., 1949. Respiratory enzymes. Burgess Publishing Co., Minneapolis.

Nelson, J. M., and C. R. Dawson, 1944. Tyrosinase. *Adv. Enzymology*, **4**: 99–150.

Potter, V. A., and C. A. Elvehjem, 1936. A modified method for the study of tissue oxidations. *J. Biol. Chem.*, **114**: 495–504.

von Brand, T., 1952. Chemical physiology of endoparasitic animals. Academic Press, New York.